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Water soluble multiarm-polyethylene glycol-betulinic acid prodrugs: design, synthesis, and *in vivo* effectiveness

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Betulinic acid (BA) is a new type of cancer-fighting drug, but it is limited by the low water solubility and relatively short half-life in clinical applications. To overcome the shortcoming, BA prodrugs were prepared by multiarm-polyethylene glycol linkers. The prodrugs exhibited high drug loading capacity (3.26 ~ 11.81 wt%), high water solubility (290 ~ 750 fold of free BA), and excellent *in vitro* anticancer activity. Subsequent tumor xenograft assays demonstrated superior therapeutic effect of BA prodrugs on inhibition of tumor growth compared with free BA. A multiple intravenous injection of BA prodrugs equivalent to 10 mg of BA/kg resulted in the decrease of an established implanted murine Lewis lung carcinoma (percent tumor growth inhibition after treatment at day 20, 72.1% ~ 90.7%) in mice. These results strongly supported that the BA prodrugs are promising for cancer therapy.

Instruction

Betulinic acid (BA) is a plant-derived pentacyclic lupine-type triterpene, which was discovered in a National Cancer Institute drug screening program of natural plant extracts, and has been recognized to possess potent pharmacological properties.[1] Interestingly, the white birch bark has a long tradition in folk medicine for treatment of stomach and intestinal problems used for example by China, native Americans and Russia.[2, 3] BA was discovered in 1995 as a potent anti-melanoma compound. It showed *in vitro* cytotoxic activity against melanoma cell lines.[1] The mechanism of action involves mitochondrial membrane permeabilization[4-6] with release of factors like cytochrome c, Smac[7] or apoptosis-inducing factor (AIF)[8] in a permeability transition pore-dependent manner, activating caspases and nuclear fragmentation. Later on, cancer cells derived from other tumor types such as breast, pancreas, brain and blood squamous cellular carcinoma cells were also discovered to be sensitive to BA.[7, 9-11]

Although BA has demonstrated an anticancer activity, it still has some drawbacks such as low water solubility and relatively short half-life,[1, 12-14] which has limited its drug efficacy. Currently, a variety of drug delivery systems such as liposomes, dendrimers, microcapsules, polymeric micelles and prodrugs have been developed to address these problems and further to promote sustained, controlled, and targeted delivery of poorly water soluble anticancer drugs.[15, 16] Of all these delivery systems, polyethylene glycol (PEG) has gained considerable attention as a versatile prodrug platform due to their capability to solubilize very insoluble molecule compounds, prolong circulation time, and alter the biodistribution in tumors through the enhanced permeability and retention (EPR) effect of parent drugs.[17, 18] Linear PEG is the most widely used in the macromolecular drugs modification because of its simple synthetic steps and high aqueous solubility.[19] However, a traditional linear PEG has only one or two functional groups available for the conjugating one or two drug molecules by covalent bonds, so the linear structure of PEG limits the loading capacity for small molecule drugs. Therefore, multiple PEG platforms are required to ensure the widest availability of small-molecule choices and the greatest variety of drug enhancements. Multiarm PEG with more functional groups and appropriate molecular weight (~40 KDa) involves a prodrug approach with the goal of increasing the drug loading, the circulating half life and extended exposure of drugs that are administered parenterally. Some studies used multiarm-PEG linkers to enhance the circulating half-life, solubility, and *in vivo* efficacy of the free anticancer drug,[20] but it is necessary to find more model drugs to extend the application of coupling prodrug to multarm-PEG.

In this study, we reported prodrugs of BA using multiarm-PEG linkers to improve the water-solubility, drug loading capability, and efficacy *in vivo* of BA. Direct conjugation of BA to multiarm-PEG through the 3-hydroxy group was accomplished using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride as coupling reagent with 4-dimethylaminopyridine as the organic base. The obtained multiarm-polyethylene glycol–betulinic acid (PEG-BA) was characterized including structure, molecular weight, loading capacity and *in vitro* drug release. Finally, the *in vitro* and *in vivo* antitumor activities of PEG-BA were also investigated. We wish to report on the development of an alternate technology, multiarm-PEG conjugation, for dissolving and delivering betulinic acid. This technology can also be applied to other insoluble anti-cancer drugs.

Experimental

Materials

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Four arm-polyethylene glycol carboxylic acid (4arm-PEG-COOH, Mw = 40 KDa), eight arm-polyethylene glycol carboxylic acid (8arm-PEG-COOH, Mw = 20 KDa, 40 KDa), were received from JenKem Technology Co., Ltd. (Beijing, China), and is an FDA and EU foodgrade material. Betulinic acid (BA) was obtained from Chengdu Preferred Biotechnology Co., Ltd (Sichuan, China). 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), were supplied by the J&K Chemical Reagent Co., Ltd. (Beijing, China). Penicillin and streptomycin, Gibco Dulbecco's Phosphate-Buffered Saline (DPBS), Gibco Roswell Park Memorial Institute 1640 medium (RPMI 1640), Gibco Dulbecco's Modified Eagle's Medium (DMEM) were all bought from Invitrogen. Fetal bovine serum (FBS) was from HyClone. Cell-Counting Kit-8 (CCK-8) kit was supplied by the Dojindo Laboratories. Ribonuclease A from bovine pancreas (RNAse A) and propidium iodide (PI) were purchased from Sigma-Aldrich.

Human lung cancer cells (A549) and murine Lewis lung carcinoma (LLC) cells were obtained from the Peking University Health Science Center (Beijing, China) and were grown in the listed medium: A549 (RPMI 1640 with 10% FBS, 1% streptomycin-penicillin and LLC (DMEM with 10% FBS, 1% streptomycin-penicillin). All cell lines were maintained in an incubator supplied with 5% CO_2/95% air humidified atmosphere at 37 °C.

Female C57BL/6 mice, 6-7 weeks age, were purchased from purchased from the National Institute for the Control of Pharmaceutical and Biological Products. All animal experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals, and approved by Experimental Animal Ethics Committee in Beijing.

Synthesis of 4arm-PEG$_{40K}$-BA, 8arm-PEG$_{40K}$-BA and 8arm-PEG$_{20K}$-BA conjugates

Fig. 1 shows the synthesis sequence of multiarm-PEG-BA conjugate. Synthesis of the conjugates is detailed below.

8arm-PEG$_{40K}$-COOH (10.0 g, 0.25 mmol) and BA (2.28 g, 5.0 mmol) were dissolved with 250 mL of THF. The solution was cooled to 0 °C and EDC (0.58 g, 3.0 mmol), DMAP (0.61 g, 5.0 mmol) were added. The mixture was stirred at 0 °C for 1 h and at room temperature overnight. The solvent was evaporated under vacuum. The residue was dissolved in 100 mL of THF, and the crude product was precipitated with ethyl ether (500 mL). After filtration, the resulting solids were recrystallized with a mixture of DMF/IPA (120 mL/480 mL). Then, the solids were filtered, washed with ethyl ether (2 × 500 mL), and dried under vacuum at 40 °C to give 8arm-PEG$_{40K}$-BA (99.08% yield, Fig. S4). 4arm-PEG$_{40K}$-BA and 8arm-PEG$_{20K}$-BA were similarly synthesized and purified as that for 8arm-PEG$_{40K}$-BA. The purity of PEG-BA conjugate was detected by high performance liquid chromatography (HPLC) (Supplementary Information).

Drug-to-carrier ratio characterization

A mass and molar ratio of drug-to-carrier for the BA prodrugs were determined as described here. BA was detected by UV-Vis spectrophotometer as reported earlier.[21] Briefly, BA was dissolved in 75 mL acetonitrile/water (80/20 w/w) solution and filtered. Then The UV absorbance of the derivative of BA was determined at 210 nm for five different concentrations ranging from 20 to 100 μg/mL. The pretreatment of BA prodrugs was the same as free BA. A mass or molar of PEG-BA (*mConjug or nConjug*) were diluted in a known concentration, and absorbance at 210 nm and the concentration of BA in the sample was used to obtain the mass and molar of BA (*mBA, nBA*). The mass and the molar ratio of drug-to-carrier were thus reported as *mBA/mConjug* and *nBA/nConjug*.

Solubility studies

The solubility of the prodrug was detected as reported earlier.[22] Briefly, excess amounts of PEG-BA were added to the screw capped scintillation vials containing 10 mL of various solvents or purified water. The suspension was mixed at ambient temperature. An aliquot of the sample (5 mL) was taken at 1 h intervals. Each withdrawn sample was diluted and filtered using a 0.45 μm PTFE filter, and then analyzed directly by HPLC as described in supplementary information.

Stability study

BA prodrugs (10 mg/mL) were diluted into phosphate buffered saline adjusted to pH 6.1, 7.3, and 8.1 and incubated at 37 °C. Aliquots were removed at different time points, high-speed centrifuged to get supernatant, pretreatment as described previously, and then analyzed by HPLC at 210 nm, measuring disappearance of the prodrugs. Stability profile graph was generated by plotting the percentage of remaining starting material over a time course at different pH values. The percentage was calculated on the basis of the absorbance of the sample at 0 to 4.5 days vs the initial area peak.

Hemolysis assay

The hemolytic activity of polymer solutions was investigated as previously described.[23, 24] Fresh blood samples were collected through cardiac puncture from rats. Ten milliliters of blood was added with EDTA-Na$_2$ immediately to prevent coagulation. The red blood cells (RBCs) were collected by centrifugation at 1500 rpm for 10 min at 4 °C. After washing in ice-cold DPBS until the supernatant was clear, erythrocytes were diluted at a final concentration of 5×10^8 cells/mL in ice-cold DPBS. 1mL BA prodrugs or PEI$_{25K}$ solution (1 mg/mL and 0.1 mg/mL) was mixed with 1 mL erythrocyte suspension. DPBS and 1% Triton X-100 in DPBS were used as controls for 0% lysis and 100% lysis, respectively. Samples were incubated for 1 h at 37 °C under constant shaking. After centrifugation at 1500 rpm for 10 min at 4 °C, supernatant was analyzed for hemoglobin release at 541 nm using an infinite M200 microplate spectrophotometer (Tecan, Switzerland). Hemoglobin release was calculated as $(OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control}) \times 100\%$. Hemolysis was determined from three independent experiments.

In vitro cell cytotoxicity

Lewis lung carcinoma cells (LLC), human lung cancer cells (A549) cell lines were supplied by Peking University Health Science Center. These lines were grown in RPMI 1640 supplemented with 10% FBS, 1% streptomycin-penicillin, and subcultured 2-3 times/week. All cells were grown in a humidified incubator at 37 °C, 5% CO_2.

CCK-8 assay was used for cell evaluation of different samples.[25, 26] LLC and A549 cells were respectively seeded at a

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density of 3×10^3 and 4×10^3 cells/well respectively in 180 µL culture medium within a 96-well plate (Corning, USA) and incubated overnight. Then, the cells were treated with various samples (BA, 4arm-PEG$_{40K}$-BA, 8arm-PEG$_{40K}$-BA, and 8arm-PEG$_{20K}$-BA) at 37 °C in a humidified incubator with 5% CO_2 for 24 h, 48h, and 72 h, where the samples of the free BA and BA prodrugs were dissolved in dimethylsulfoxide (Merck, Darmstadt, Germany) and diluted into tissue culture medium before assay and BA dose ranged from 2.5 to 500 µg/mL. 20 µL of CCK-8 solution was added to each well of the plate and incubated for another 1 h at 37 °C. The absorbance at 450 nm was measured by infinite M200 microplate spectrophotometer. Percent viability was normalized to cell viability in the absence of the samples. The IC_{50} was calculated as the prodrug concentrations which inhibited growth of 50% of cells relative to non-treated cells according to Unger et al.[27] IC_{50} was calculated using the Boltzmann sigmoidal function from Origin® 8.6 (OriginLab, Northampton, USA). Data are representative of three independent experiments.

Apoptotic cells were detected using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, BD Biosciences, Heidelberg, Germany). 4.0×10^5 LLC cells were treated with 8arm-PEG$_{40K}$-BA conjugates at their IC_{80} values during 48 and 72 h. Adherent and floating cells were harvested, centrifuged (1000 rpm, 3 min, 4 °C) and washed twice with 1 mL PBS (with Ca^{2+}/Mg^{2+}). The cells were resuspended in 400 µL binding buffer (1×). Each sample was treated with 5 µL Annexin V-FITC, and kept in the dark for 15 min, then 5 µL propidium iodide (PI) was added, and kept in the dark for 5 min, 0 °C. 400 mL binding buffer (1×) was added to each sample and they were analyzed by flow cytometry, using about 1×10^4 events.

In vivo efficacy studies

Subcutaneous tumor xenograft models were established in the right axillary flank region of female C57BL/6 mice (6-7 wk) by injecting 1×10^6 LLC cells per mouse. Treatment with BA or BA prodrugs given as multiple doses of 50 mg/kg (every 2 day, q2d × 5) was initiated when tumors reached an average volume of 100 to 150 mm^3. It is important to note that the doses or concentrations of BA prodrugs stated in this article refer to BA equivalents. For example, a dose of 25 mg/kg of 8arm-PEG$_{40K}$-BA means that the dose contains 25 mg/kg of BA and 725 mg/kg (29-fold higher) of whole prodrug, here the loading of BA in the whole 8arm-PEG$_{40K}$-BA is 6.67%. In the observation phase, mice were monitored for tumor sizes and body weights every other day. Tumor volume was calculated using the formula: $(L \times W^2)/2$, where L is the longest and W is the shortest tumor diameter (millimeter). Relative tumor volume (RTV) was calculated at each measurement time point (where RTV was equal to the tumor volume at a given time point divided by the tumor volume prior to initial treatment). For efficacy studies, the percentage of tumor growth inhibition (%TGI) was calculated using the following formula: $[(C - T) / C] \times 100\%$, where C is the mean tumor volume of the control group at a specified time and T is the mean tumor volume of the treatment group at the same time. To monitor potential toxicity, we measured the weight of each mouse. For humane reasons, animals were killed and regarded as dead if the implanted tumor volume reached to 5000 mm^3 or at the end of the experiment (> 6 wk). 200 µL of blood of each mouse were collected after final administration to further evaluate the hematological toxicity of different BA prodrugs. Obtained blood was immediately evaluated to test the white blood cell number (WBC) using a hematology analyzer (MEK-7222K, Japan).

Detection of allergic reaction

Toxic side-effects of the current chemotherapeutical drugs are often causing a severe reduction in the quality of life, so the detection of allergic reaction is very necessary and important. Five groups of tumor bearing mice (26−28 g, n = 6) were used in allergy testing studies (control, BA, 4arm-PEG$_{40K}$-BA, 8arm-PEG$_{40K}$-BA, and 8arm-PEG$_{20K}$-BA). The four samples were administrated via tail intravenous injection every two days at the BA dose of 10 mg/kg body weight. After administration with different samples for 10 days, orbit blood of mice in different groups was collected and centrifuged. Serum samples were analyzed according to the procedure of Mouse IgE ELISA.

Statistical analysis

All experiments in this study were performed at least three times, and the data were expressed as the means standard deviation (SD). Statistical analyses were performed by analysis of variance (ANOVA). In all analyses, $p < 0.05$ was taken to indicate statistical significance.

Results and discussion

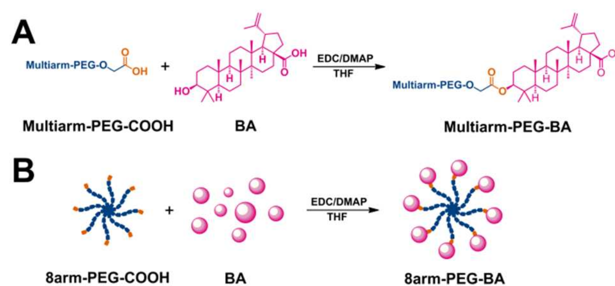

Fig. 1 Synthesis of multiarm-PEG-BA.

Synthesis of 4arm-PEG$_{40K}$-BA, 8arm-PEG$_{40K}$-BA and 8arm-PEG$_{20K}$-BA conjugates

Linear PEG is the most widely used for its simple synthetic steps and good water solubility. But the limitation of this system is its low drug loading capability. Thus, multiarm-PEG, as modified BA structures, was selected for highly efficient incorporation of drugs on the polymer scaffold. Multiarm-PEG-BA conjugates were synthesized via an esterification reaction between the carboxy groups of multiarm-PEG-COOH and the hydroxyl group of BA (Fig. 1A and 1B). The resulting PEG-BA is a white powdery solid, readily soluble in H_2O, THF, and MeCN, with no gelling properties. The chemical construction and molecular weight of BA prodrugs was measured by ^{1}H-NMR and MALDI-TOF (Fig. S1 and S2).

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Table 1. Drug/carrier ratio, Solubility and Hydrolysis Half-lives of BA prodrugs

compound	Drug/carrier ratio		solubility (mg/mL)	Hydrolysis $t_{1/2}$ (h)[b]		
	mass (%)	molar		pH 8.1	pH 7.4	pH 6.1
BA	--	--	0.021	--	--	--
4arm-PEG$_{40K}$-BA	3.26 ± 0.23	2.95 ± 0.20	186.4 (6.08 [a])	21.0	38.4	144.3
8arm-PEG$_{40K}$-BA	6.89 ± 0.30	6.48 ± 0.26	135.3 (9.02)	25.8	43.2	165.6
8arm-PEG$_{20K}$-BA	11.81 ± 0.50	5.86 ± 0.22	160.2 (15.78)	30.3	46.2	191.7

a Equivalent to free BA. [b] Based on the release of BA.

Absorbance properties of BA and PEG-BA

The absorbance spectra of BA and PEG-BA were measured by UV-Vis spectrophotometer (Fig. 2). The UV absorbance of free BA in acetonitrile was determined at 210 nm for five different concentrations ranging from 20 to 100 µg/mL. From the standard plot of concentration vs absorbance, the coefficient (Δ) for BA was calculated to be 77.58 µg/mL. The BA prodrugs were dissolved in acetonitrile at an approximate concentration of 1 mg/mL (based on M_W of 20 or 40 kDa), and the UV absorbance of them at 210 nm was determined. Using this value, and employing the coefficient Δ obtained from above, the concentration of BA in the sample was determined. Thus, dividing this value by the PEG-BA concentration provided the percentage of BA in the prodrugs (Table 1). The mean mass ration of drug-to-carrier for 4arm-PEG$_{40K}$-BA, 8arm-PEG$_{40K}$-BA, and 8arm-PEG$_{20K}$-BA was 3.26 ± 0.23, 6.89 ± 0.30 and 11.81 ± 0.50; and the molar ration of them was 2.95 ± 0.20, 6.48 ± 0.26, and 5.86 ± 0.22, respectively. The mass/molar ration indicates that approximately 1 or 2 functional groups of multiarm-PEG remained unconjugated.

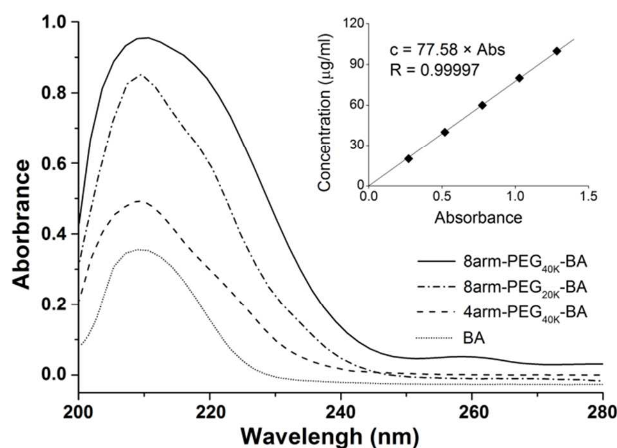

Fig. 2 Absorbance spectrum of BA and BA prodrugs in UV-Vis buffer. (Inset) Linear regression fit of BA standards to calculate the concentration for BA (n = 3 tests, 4 scans per test, R = 0.99997).

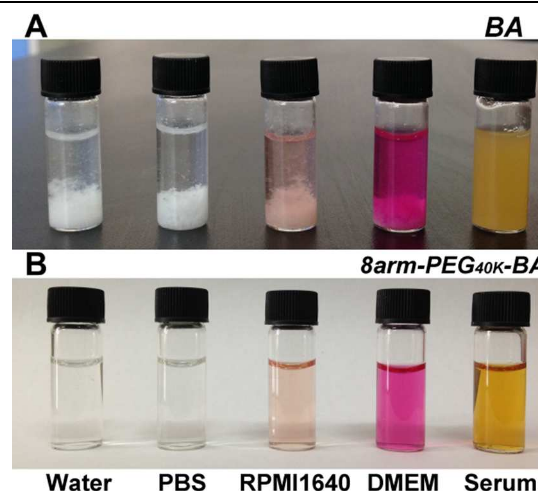

Fig. 3 BA (A) and 8arm-PEG$_{40K}$-BA (B) in different solutions recorded after centrifugation.

Solubility studies

Solubility studies were conducted to investigate the solubility of BA and BA prodrugs in various solvents (Fig. 3). As predicted, PEGylation has substantially increased the solubility of BA, which is an insoluble molecule by itself. The solubility of BA in distilled water published by Jäger et al.[21] is approximately 0.021 mg/mL. All the BA prodrugs have 160.2 to 186.4 mg/mL solubility in water, which is equivalent to as high as 15.78 mg/mL of BA (Table 1). This highly increased solubility makes it possible to systemically evaluate the therapeutic efficacy of BA *in vivo*.

Stability study

As the BA prodrugs were synthesized through covalent bonds between the carboxylic group of PEG and hydroxide radicals of free BA, we expected the hydrolytic cleavage of ester bonds to occur before the drug can exert a significant cytotoxic effect.[28] Hydrolysis studies demonstrated that free BA is released from PEG-BA in PBS and the hydrolysis rate is strongly dependent on pH. BA prodrugs were placed in aqueous solutions that simulated biological fluids to measure the rates of hydrolysis by UV-Vis analysis. The stabilities of the BA prodrugs were determined in buffered solutions at pH 8.1 (Fig. 4A, Fig. S6), pH 7.4 (Fig. 4B, Fig. S7), and pH 6.1 (Fig. 4C, Fig. S8) at 37 °C to simulate the blood environment.[29, 30] In the preclinical studies, the release of

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BA could be due to several

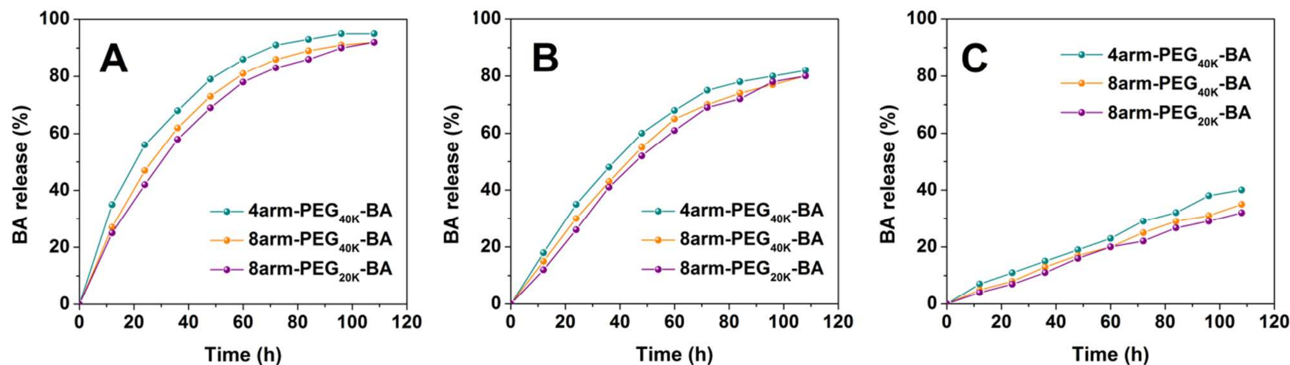

Fig. 4 Stability of BA prodrugs in water at 37 °C. Experiments were respectively done at pH 8.1 (A), at pH 7.4 (B), and at pH 6.1 (C) for 4arm-PEG$_{40K}$-BA, 8arm-PEG$_{40K}$-BA, and 8arm-PEG$_{20K}$-BA. The presence of free BA was monitored as a function of time.

mechanisms as previously proposed.[31, 32] The resultant hydrolysis data is shown in Fig. 4 and the half-lives for these curves are given in Table 1. All prodrugs were much more stable at pH 6.1 than at pH 7.4 or 8.1, and the stability trend was the same as the trend of hydrolysis rates for the three prodrugs (8arm-PEG$_{20K}$-BA<8arm-PEG$_{40K}$-BA<4arm-PEG$_{40K}$-BA). The hydrolysis half-lives of the prodrugs were increased 3-fold and 5-fold at pH 6.1 over pH 7.4 and pH 8.1 values, respectively.

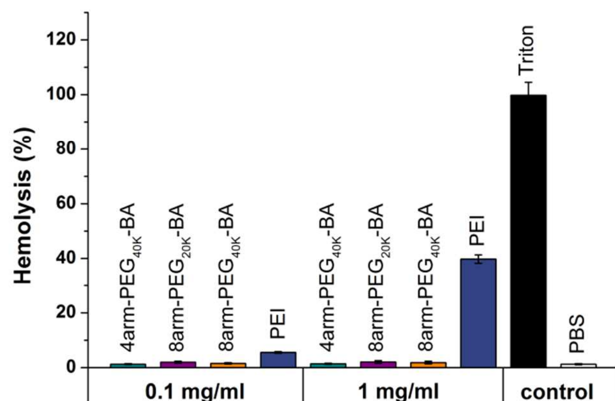

Fig. 5 Hemolysis assay of BA prodrugs compared to PEI$_{25K}$ and Triton X-100 measured at 541 nm (error bars represent standard deviation).

Hemolysis study

The detrimental interactions between prodrugs and blood constituents such as RBCs must be avoided when injecting the prodrugs into the blood circulation as a carrier for drug delivery.[24] Erythrocytes were incubated with two concentrations of polymer as 1 mg/mL and 0.1 mg/mL, for 1 h at 37 °C. Hemolysis was evaluated by measuring the amount of hemoglobin released in the supernatant at 541 nm (Fig. 5), and Triton X-100 was used as positive control, which induced full hemoglobin release. All of BA prodrugs at concentrations of 1 mg/mL and 0.1 mg/mL showed a comparable hemoglobin release to blank values (<2%), which was significantly lower than comparable concentrations of PEI$_{25K}$, a cationic polymer known to have a significant hemolytic effect.[24] Despite free BA was cytotoxic to the RBCs in a previous study,[33] BA prodrugs have been hydrolyzed a little in the short incubation period (approximately 1 h), suggesting the excellent safety.

***In vitro* cytotoxicity**

To ensure the effectiveness of the prodrug before their entry into human application, *in vitro* cytotoxicity should be considered upfront.[34, 35] To examine the cytotoxicity of free BA and BA prodrugs, a CCK-8 assay was conducted after incubating cells with different treated. The response of two cells (A549 and LLC) was tested *in vitro* by seeding the cells and exposing them to various concentrations of BA prodrugs and free BA. Cells were exposed to drug for 24, 48 or 72 h. Analysis of *in vitro* cytotoxicity measurements showed that free BA applied at 500 μg/mL induced cell death which was dependent upon the length of incubation, starting with approximately 25% cell viability after 24 h and leading to less than 10% cell viability after 3 days of incubation. BA prodrugs also have the same trend. Representative data for BA prodrugs and free BA appear in Fig. 6A and 6B. At a dose of 500 μg/mL PEG-BA prodrugs, the viability was even reduced by ~85% and ~90% for LLC and A549 cells, respectively. BA prodrugs were equipotent to free BA.

To compare the potency of prodrugs, the concentrations of drug which killed 50% of the cells (IC_{50}) were estimated from survival curves, including the curves as show in Fig. 6C and 6D, obtained from replicate experiments. The IC_{50} of BA prodrugs were slightly greater than free drug (Table 2). All prodrugs were sensitivity of cells with the trend in the IC_{50} values for the samples remaining the same (4arm-PEG$_{40K}$-BA > 8arm-PEG$_{40K}$-BA > 8arm-PEG$_{20K}$-BA > BA). These values are virtually equivalent to that for the free drug, indicating that BA is being released into the medium. Also, the IC_{50} values of prodrugs correlated with the hydrolytic stabilities of the compounds in PBS, indicated that more prodrugs were needed to kill an equivalent fraction of cells, because the prodrugs released BA over time whereas incubation with free drug resulted in a bolus dose. *In vitro* experiments the tumor cell culture does not capture the advantages of PEGylated BA compared to free BA, such as improved pharmacokinetics, and hence may underestimate the efficacy of BA prodrugs.

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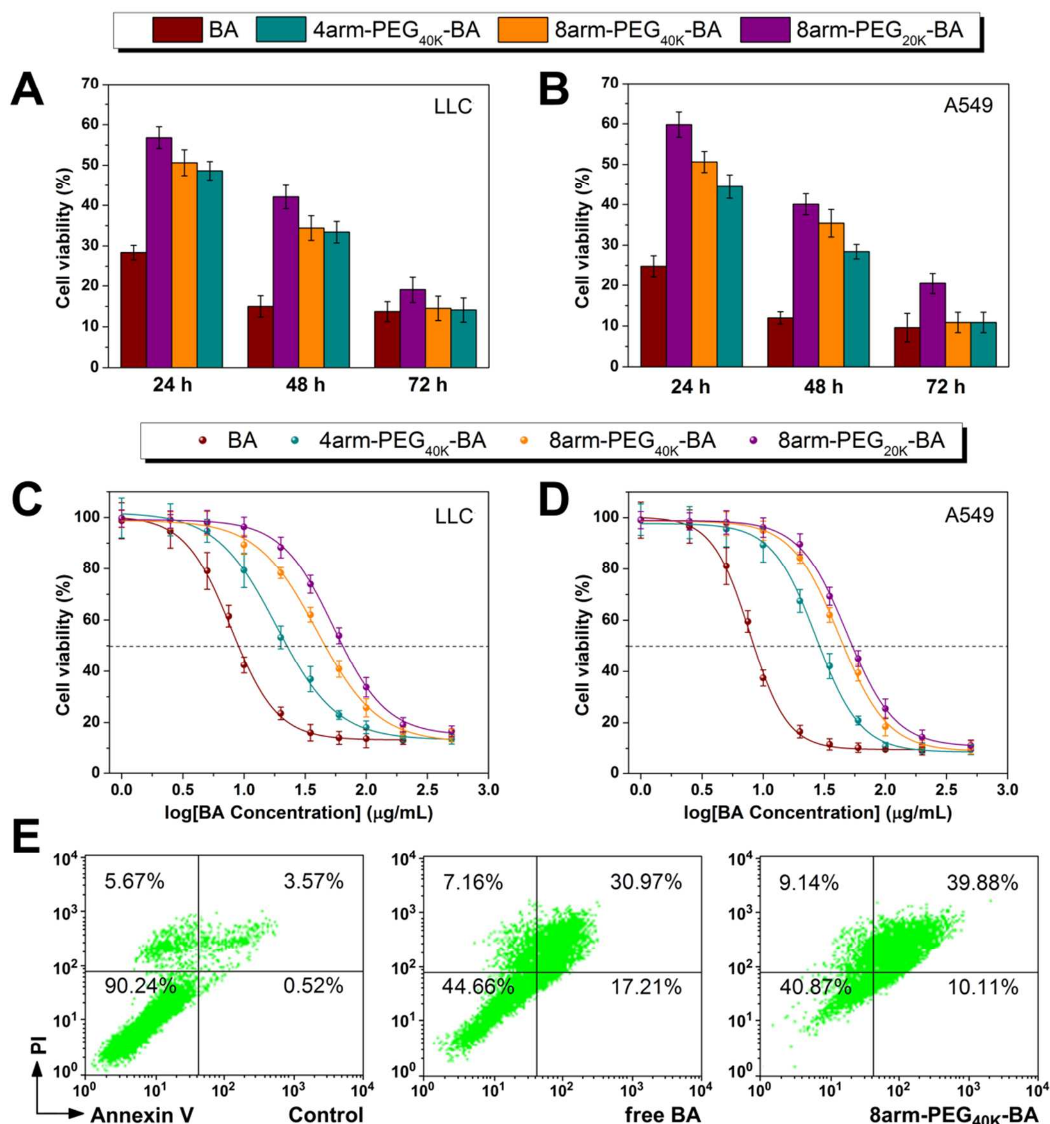

Fig. 6 Cellular cytotoxicity of free BA and BA prodrugs in A549 and LLC cells. Cell viability of LLC (A) and A549 (B) cells treated with 500 μg/mL of BA and PEG-BA (equivalent to free BA) was measured by CCK-8 assay (n = 3, error bars represent standard deviation). CCK-8 assay of BA and BA prodrugs with different concentration in LLC (C) and A549 (D) cell lines (n = 3, error bars represent standard deviation). (E) Flow cytometry analysis for apoptotic and necrotic LLC cells stained with Annexin V-FITC (AV) and propidium iodide (PI) after treatment with IC_{50} of BA or 8arm-PEG$_{40K}$-BA for 72 h. Normal viable cells (AV-/PI-) are shown in the *bottom left window*, Early apoptotic cells (AV+/PI-) in the *bottom right window*, and the population of terminal apoptotic/necrotic cells (AV+/PI+) in the *top right window*, secondary necrosis (AV+/PI+) in the *top left window*. Sizes of cell sub-populations are given as percentage of total populations.

Table 2. *In Vitro* Cytotoxicity of BA prodrugs (IC_{50}, μg/mL)

compound	LLC	A549
BA	7.16 (0.49935)	7.78 (0.23578)
4arm-PEG$_{40K}$-BA	37.03 (0.50748)	27.19 (1.1777)
8arm-PEG$_{40K}$-BA	47.65 (0.61244)	39.57 (0.92405)
8arm-PEG$_{20K}$-BA	58.15 (0.60601)	44.18 (0.94063)

We further evaluated the apoptotic effect of BA to distinguish it from necrotic effect. It can be also used to quantify three populations of cells with drug treatment: normal viable cells, apoptotic cells, and populations of necrosis using Annexin V-FITC and PI staining. It was observed in Fig. 6E, normal viable cells (AV-/PI-) are shown in the *bottom left window*, Early apoptotic cells (AV+/PI-) in the *bottom right window*, and the population of terminal apoptotic/necrotic cells (AV+/PI+) in the

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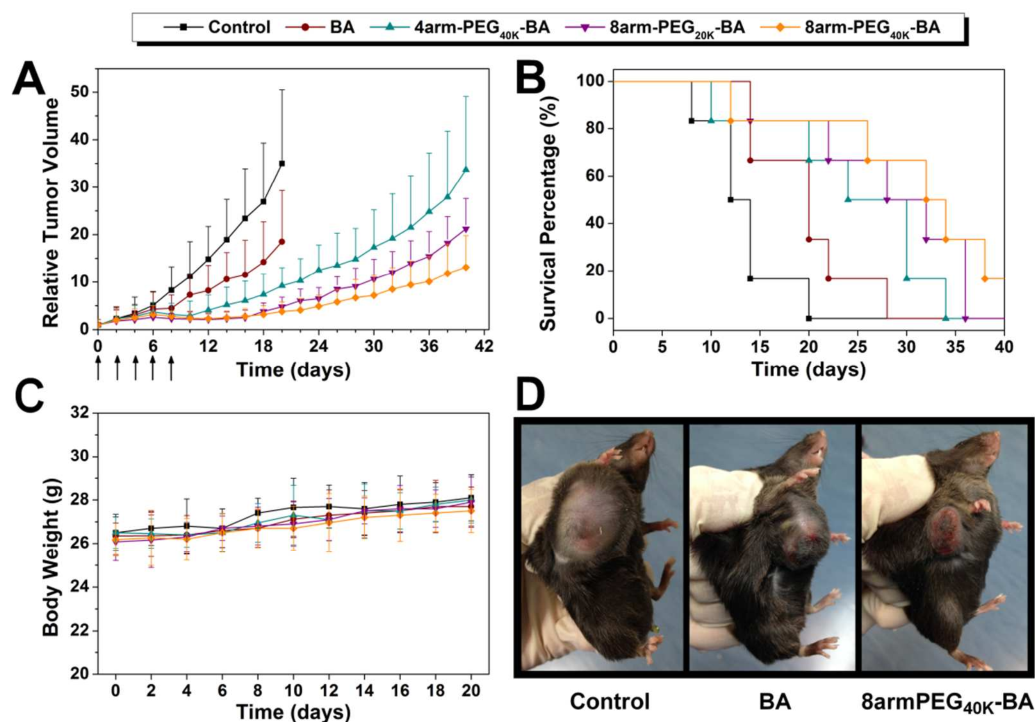
Table 3. LLC Xenograft Model (10 mg/kg q2d × 5): Efficacy Comparison

compound	mean TV±SD (mm^3)[a]	RTV[a]	TGI(%)[a]	Cures(%)[b]
control	4450 ± 1900	35.6 ± 15.2	0	0
BA	2368 ± 1382	18.5 ± 10.8	46.7	16.7
4arm-PEG$_{40K}$-BA	1242 ± 437	10.8 ± 3.8	72.1	50.0
8arm-PEG$_{20K}$-BA	561 ± 234	4.8 ± 2.0	87.4	66.7
8arm-PEG$_{40K}$-BA	414 ± 261	3.8 ± 2.4	90.7	83.3

[a] Mean tumor volume (TV), RTV, and % TGI data were taken at day 20. (By day 20, a significant percentage of control animals were euthanized due to excess tumor burden.) [b] % cures were taken at day 26.



5 **Fig. 7** Antitumor efficacy of BA and different BA prodrugs in the subcutaneous mouse model of LLC. (A) Tumor volumes of mice during treatment with different BA prodrugs. (B) Survival of mice in different treatment groups. (C) Body weight after administration. (D) Tumor images of different groups after treatment at day 20.

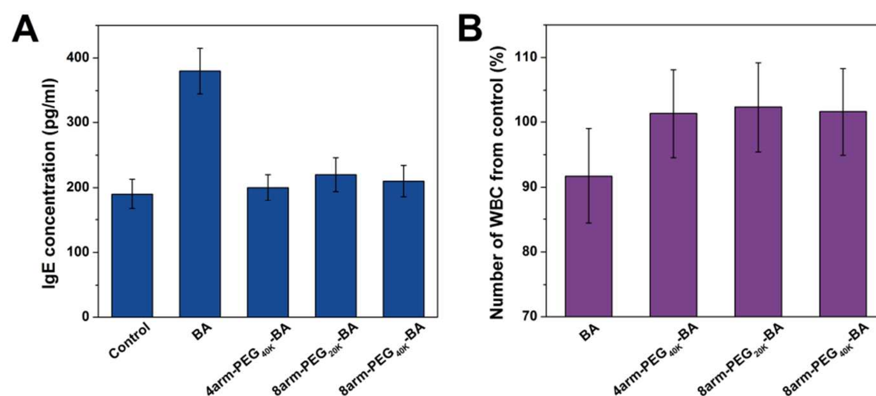


Fig. 8 Caption Subacute toxicities of different groups were reflected by IgE levels (A) and the WBC change (B) of mice. Data as means ± S.E.; $n = 6$.

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top right window, secondary necrosis (AV+/PI+) in the *top left window*. Untreated LLC cells yielded only 0.52% apoptotic cells and 9.24% necrosis cells; LLC cells treated with BA (IC_{50}) for 72 h showed 17.2% apoptotic cells and 38.13% necrosis cells; and LLC cells treated with 8arm-PEG_{40K}-BA (IC_{50}) for 72 h showed 10.11% apoptotic cells and 49.02% necrosis cells. This observation is in agreement with the cytotoxicity results.

In vivo studies

The results described above gave us great confidence to evaluate the anticancer effectiveness of BA formulations in a mouse tumor model. The efficacies of BA prodrugs were compared with free BA at equivalent doses of 10 mg/kg BA on multiple-dose schedule in xenograft models of lung tumor. For humane reasons, animals were killed and regarded as dead if the implanted tumor volume reached 5000 mm^3 or at the end of the experiment (> 6 wk). The two most important goals in cancer treatment are prolonged survival without reduction in the quality of life. Tumor-bearing mice treated with BA prodrugs showed a clear survival advantage compared with the control treated mice (Fig. 7B). As shown in Fig. 7A, the groups treated with BA and different BA prodrugs showed varied levels of antitumor effects and they were ranked as 8arm-PEG_{40K}-BA > 8arm-PEG_{20K}-BA > 4arm-PEG_{40K}-BA > BA. The treatment with 8arm-PEG_{40K}-BA resulted in 90.7% TGI (day 20) and 83.3% survival of animals (day 26). In contrast, multiple-dose BA treatment resulted in 46.7% TGI (day 20) and 16.7% survival of animals (day 26) (Fig. 7A and 7B, Table 3). Importantly, in line with the literature, no signs of systemic toxicity were observed by monitoring general behavior, appetite and mice body weight (Fig. 7C).

As expected, tumor growth of mice was significantly inhibited by BA prodrugs because of the appropriate molecular weight and good bioavailability. The reason could be that the prolonged tumor residence time and sustained drug release offer sufficient opportunities for BA to be attained at the tumor site through the EPR effect. As a result, 8arm-PEG_{40K}-BA conjugate could maintain an effective therapeutic concentration for a long period of time through the depot effect and thus had an increased therapeutic effect. Simultaneously, the median survival time was significantly extended after treatment with the 8arm-PEG_{40K}-BA conjugate in comparison with other groups which may due to the appropriate molecular weight (~ 40 KDa) and high drug loading (~ 6.89 wt%). The tumors of the mice treated with PBS, PEG, BA, and different BA prodrugs were strong evidence of efficacy.

Evaluation of the side effects

Although BA prodrugs showed significant therapeutic effects *in vivo*, whether these prodrugs had non-negligible adverse effects remained a critical issue. During the early development of the drugs, type I hypersensitivity is the most common type of the hypersensitivity reaction. Some of the natural anti-cancer drugs, such as paclitaxel, docetaxel, and teniposide cyclosporine, were usually associated with a high incidence of the type I hypersensitivity reaction. It has been demonstrated that IgE antibodies play an important part in mediating type I hypersensitivity responses. We thus selected IgE levels as the parameter for rapid evaluation of type I hypersensitivity reactions. The blood IgE levels of mice in different groups (BA, 4arm-PEG_{40K}-BA, 8arm-PEG_{20K}-BA, and 8arm-PEG_{40K}-BA) were shown in Fig. 8A. Mice treated with BA displayed a higher IgE level than the control group, which might be ascribed to the bad water solubility. As expected, no significant change of IgE level was observed in the BA prodrugs groups, which explored the idea that the use of these prodrugs could reduce the risk of hypersensitivity reactions substantially. The blood of mice after treatment with different BA formulations was also collected to test the WBC count, which is often used as an indicator of hematologic toxicity. The total WBC count of mice treated with free BA showed a little decrease over the normal group (Fig. 8B). No discernible decreases in WBC number of the mice treated with BA prodrugs were observed, indicating that the BA prodrugs designed in this study could avoid severe hematotoxicity.

Conclusions

PEGylation strategy is a promising approach to improve pharmaceutical properties of small molecular drugs and to enhance the efficacy due to the EPR effect. Using multiarm-PEG linkers, we successfully developed several water-soluble BA prodrugs that enable significantly increased solubility, parenteral delivery of BA, and significantly enhanced therapeutic index in lung xenograft models. The chemistry for the BA prodrugs ensures that the anticancer molecule can be released from the prodrugs. In addition, the white blood cell count in the mice, as an indication of hematopoietic toxicity, was not affected after i.v. BA-treatment. Together, these results indicate that BA prodrugs have the potential to slow the outgrowth of tumors from lung carcinomas, thereby prolonging life, without inducing systemic adverse effects. Of the three different BA prodrugs, 8arm-PEG_{40K}-BA has high drug/carrier ratio, retains the biological activity well *in vitro*. *In vivo*, 8arm-PEG_{40K}-BA conjugate shows antitumor activity that is more potent than that of BA or other BA prodrugs. Therefore 8arm-PEG_{40K}-BA was selected as the lead candidate for further preclinical development.

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Notes and references

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† Electronic Supplementary Information (ESI) available: ^{1}H-NMR and MALDI-TOF of 4arm-PEG$_{40K}$-BA, 8arm-PEG$_{40K}$-BA, and 8arm-PEG$_{20K}$-BA conjugates and pH stability studies for them. See DOI: 10.1039/b000000x/

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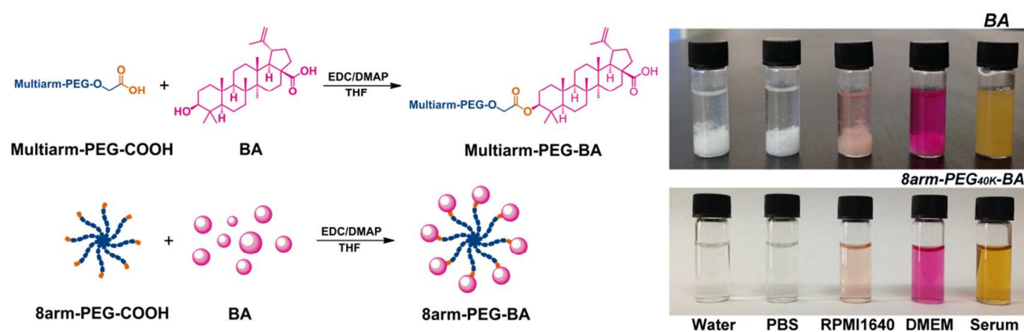
Graphical Abstract

Multiarm-polyethylene glycol-betulinic acid prodrugs were prepared by multiarm-polyethylene glycol linkers and betulinic acid, which exhibited high drug loading capacity, good water solubility, and excellent anticancer activity.



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